pH Sensitive Coiled Coils: A Strategy for Enhanced Liposomal Drug Delivery

Rahi M. Reja,[a] Mohsina Khan,[b] Sumeet K. Singh,[a] Rajkumar Misra,[a] Anjali Shiras,[b*] Hosahudya N. Gopi[a*]

[a] Department of Chemistry, Indian Institute of Science Education and Research, Dr. Homi Bhabha Road, Pune -411 008, India; Email: hn.gopi@iiserpune.ac.in

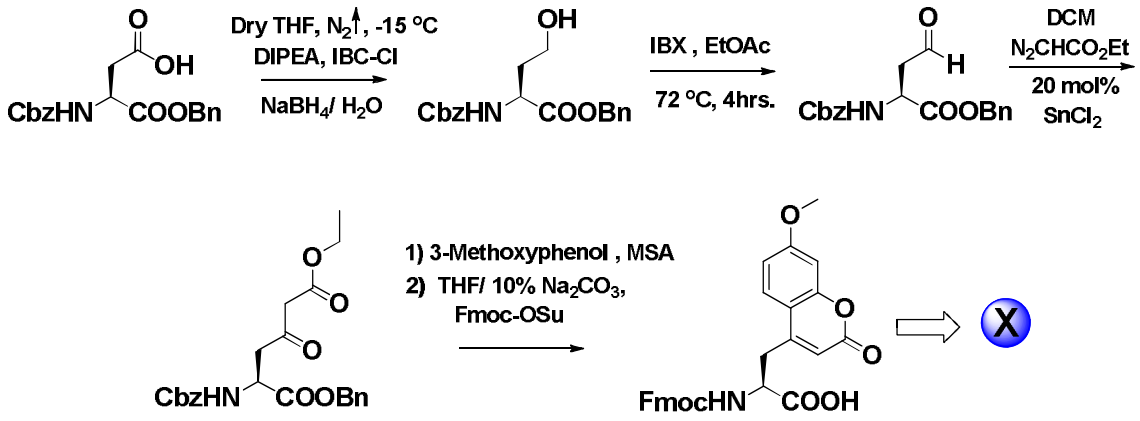
[b] National Center for Cell Sciences, Pune University Campus, Pune-411 007, India; Email: anjali@nccs.res.in

Table of Contents

1. Synthesis of the Fmoc-Asp(Coumarin)-OH:[1]

N-Fmoc-Asp coumarin amino acid was synthesized starting from Cbz protected γ-amino-β-keto-esters as reported earlier. Briefly, the Cbz protected γ-amino-β-keto-esters (0.854 g, 2 mmol) was treated with 3-methoxyphenol (1.23 g, 10 mmol) and the mixture was cooled to 0 °C under N_2 atmosphere. After stirring for 5 min, MSA (4.8 g, 50 mmol) was added slowly under constant stirring. The reaction mixture was allowed to come to room temperature. The reaction was monitored by TLC. After completion of the reaction (approximately 2 hrs), the reaction mixture was diluted with diethyl ether (200 mL) and cooled to -15 °C. The free amino acid was separated as red precipitate (methanesulfonate salt of coumarin) after centrifugation (4000 rpm at 4 °C) and subjected for Fmoc-protection using Fmoc-OSu as described below. The free amino acid was dissolved in a solution of 30% Na_2CO_3 (20 mL) and THF (10 mL) and cooled to 0 °C. A solution of Fmoc-OSu (0.740 g, 2.2 mmol) in THF (10 mL) was added to the reaction mixture and allowed to stir overnight to complete the reaction. After completion (monitored by TLC), the reaction mixture was washed with ether (20 mL x 2) and the aqueous layer was acidified with 3N HCl (up to pH 3) under ice cold conditions. The combined organic layer was washed with 5% HCl (50 mL x 2), brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give reddish gummy crude product which was further purified on silica gel column chromatography to give pure white powder, which was further used for the solid phase peptide synthesis. Yield: 0.508 g (50%).

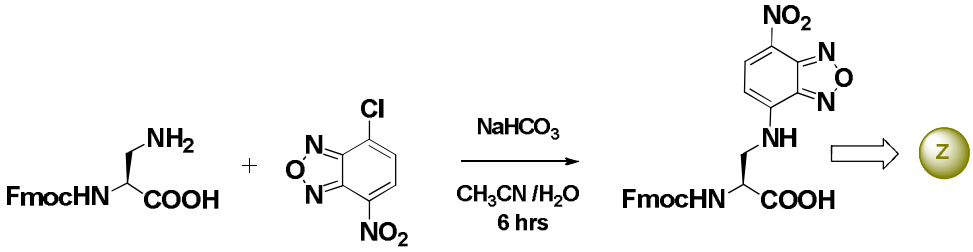
UV absorption (λ_{max}) at 324nm,Fluorescence Emmision (λ_{max}) at 384nm , **^{1}H NMR** (400 MHz, DMSO) **:**δ_H 13.09 (s, b, 1H, -COO*H*), 7.88-7.86 (d, *J* = 7.32, 2H, aromatic Fmoc), 7.74-7.63 (d, *J* = 9.16, 1H, aromatic ring of coumarin), 7.63-7.59 (dd, *J* = 7.36, *J* = 3.64 2H, aromatic Fmoc), 7.41-7.37 (t, *J*= 7.56, 2H, aromatic Fmoc), 7.31-7.25 (dd, *J* = 7.32, *J*= 6.88, 2H, aromatic Fmoc), 7.02-6.979 (m, 2H, aromatic ring of coumarin), 6.24 (s, 1H, C=C*H*), 4.33-4.27 (m, 1H, Fmoc-C*H*), 4.23 - 4.14 (m, 3H, Fmoc-CH_2), 3.84 (s, 3H, -OCH_3), 3.34-3.04 (m, 2H, CH-CH_2) **^{13}C NMR** (100 MHz, DMSO) **:**δ_C 172.58, 162.38, 159.95, 155.94, 155.04, 152.62, 143.64, 140.66, 128.16, 127.64, 127.04, 125.83, 125.12, 120.12, 112.41, 112.03, 101.13, 65.74, 56.06, 52.76, 46.51, 32.60



Scheme 1: Synthesis of the Fmoc-Asp(Coumarin)-OH through von pechmann condensation

2. Synthesis of Fmoc-Dap(NBD)-OH fluorescent amino acid:[2]

To a stirred solution of Fmoc-L-diaminopropionic acid (0.5 g, 1.53 mmol) in water /acetonitrile (20 mL of a 1:1 mixture) was added 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, 0.367 g, 1.836 mmol) and sodium bicarbonate (0.154 g, 1.83 mmol) and pH was adjusted between 8-9. The reaction mixture was stirred for 6 h at RT. Then 10 mL water was added and the mixture was washed with ethyl acetate/hexanes (1:5) twice. The aqueous phase was acidified by 10% HCl until a pH of 5–6 was achieved. The product was extracted from the aqueous solution with ethyl acetate. The combined organic phases were dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. Then it was precipitated using hexane and used for the solid phase synthesis without further purification. Yield: 0.733 g (81%) R_f = 0.37 (CH_2Cl_2: MeOH = 5:1). HRMS (ESI+): m/z calcd. for $C_{24}H_{19}N_5O_7$ $[M + H]^+$=490.1362, found 490.1371.



Scheme 2: Synthesis of NBD amino acids through S_NAr reaction

3. Fluorescence Spectroscopy:

All the fluorescence studies of the peptides were studied in 10mM PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) solution individually or with their coiled coil partners. Fluorescence was measured using excitation wavelength of particular fluorescent peptide (For NBD modified peptide P5 excitation at 466 nm and Emission wavelength 480 to 600 nm). Spectra were taken at room temperature using a 1 cm path length cuvette, an increment of 1 nm, and a 15 sec averaging time. The pH dependent fluorescence study was carried out at 2.8-10.8 pH range. The acidic pH was achieved by adding dilute AcOH and basic pH was achieved by dil K_2CO_3 solution. The pH of the solution was measured by pH meter.

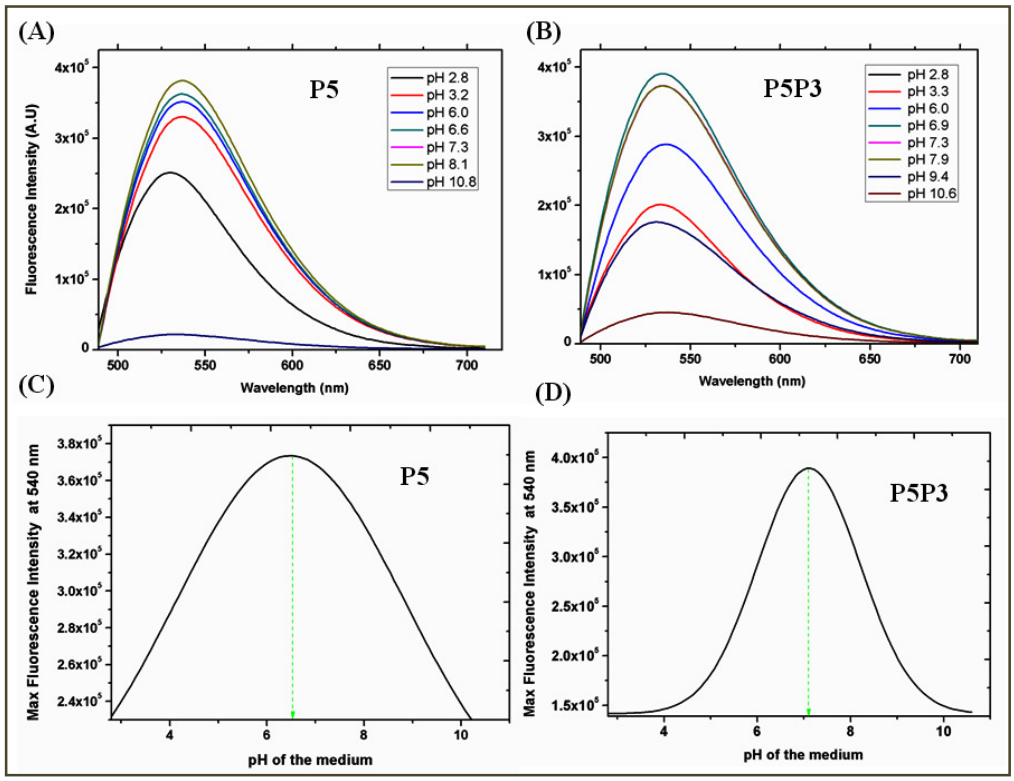


Figure S1:(A) and (B) are the fluorescence emission spectra of the peptide **P5** and **P3P5**(C) and (D) are the change in the fluorescence intensity at λ_{em}=540 nm of the peptide **P5** and **P3P5** with change in the pH of the medium.

4. Circular Dichroism (CD) Spectroscopy:

Temperature and pH dependent circular dichroism spectra were recorded using CD spectropolarimeter fitted with a peltier temperature controller. The CD spectra were measured at 30 µM total peptide concentration (15 µM each) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) at 20 °C in 2 mm quartz cuvettes at 50 nm/min scanning speed. Thermal-denaturation experiments were performed by heating from 20 to 60 °C at a rate of 1 °C/min. The CD signal at 222 nm was recorded at 5 °C intervals. For the pH dependent circular dichroism, acidic pH was achieved by adding dilute AcOH and basic pH was achieved by the dil K_2CO_3 solution. All experiments were performed in triplicate**.**

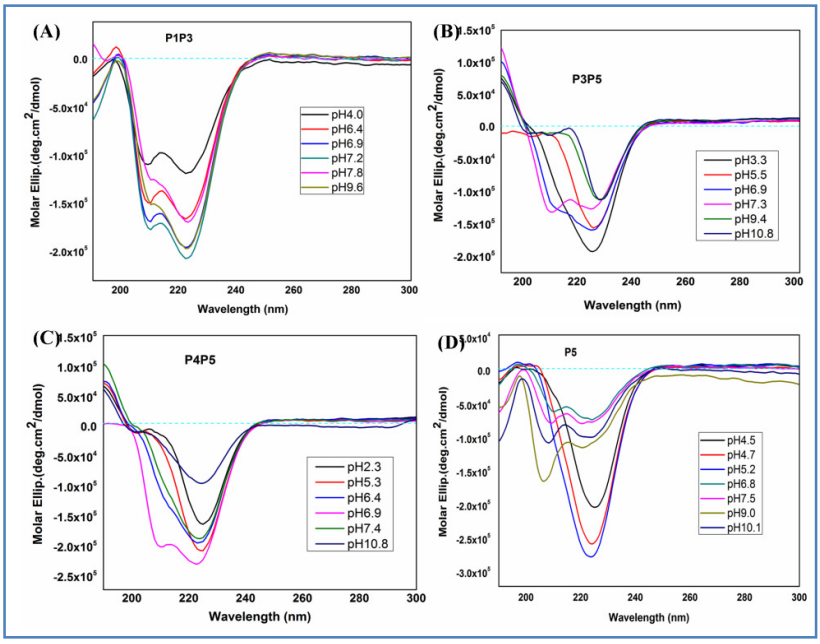


Figure S2: Circular dichroism spectra of (A) **P1P3** (B) **P3P5** and (C) **P4P5** and (D) **P5** at different pH at 20°C

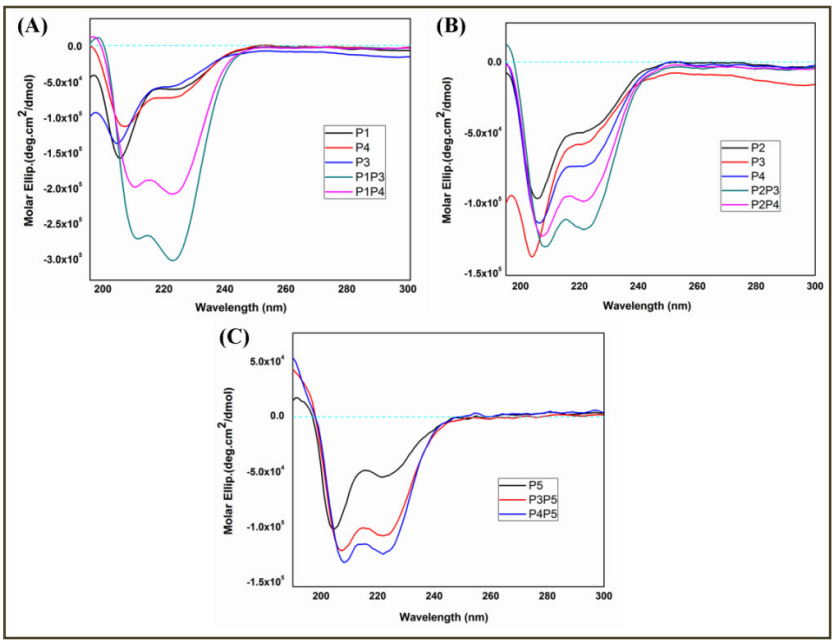


Figure S3: Circular dichroism spectra of (A) **P1**, **P4**, **P3**, **P1P3**, **P1P4** (B) **P2**, **P3**, **P4**, **P2P3**, **P2P4** and (C) **P5**, **P3P5**, **P4P5** at phosphate buffer pH 7.4 at 20 ℃

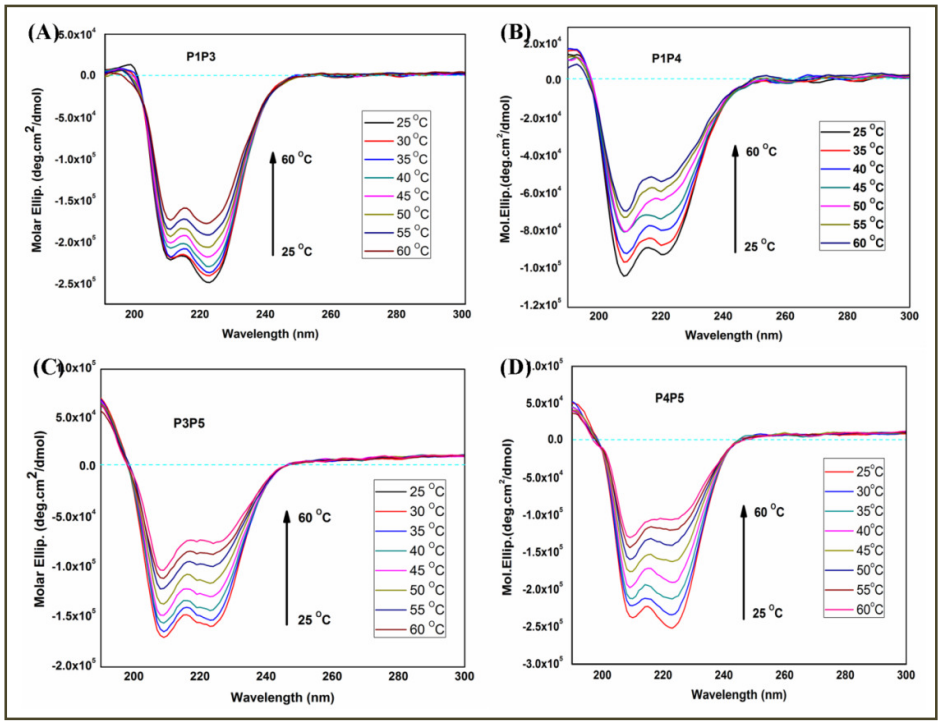


Figure S4: Temperature dependent circular dichroism spectra of the peptides with their coiled coil partners. Conc. of the each peptide is 15μM.

5. Thermodynamic Parameter Measurements from Circular Dichroism Thermal Denaturation Analysis:[3]

Thermodynamic parameters were determined by nonlinear least square fitting of the normalized CD melting curves to five parameters (a, $[\theta]_M$ (0), $[\theta]_D(0)$, ΔH_m, and T_m). Ellipticity was normalized to fraction monomer using the equation (1).

$$\theta = (\theta_M - \theta_D)f_M + \theta_D \qquad (1)$$

whereθ_M and θ_D represent the ellipticity values for the fully unfolded monomer and fully folded dimer species respectively at each temperature. θ_M was found to be constant at the temperatures higher than the melting region for all the pepetides studied. θ_D was approximated by a linear function of temperature $\theta_D = \theta_D[0] + aT$. The fraction monomer ($f_M$) was expressed in terms of the equilibrium constant after solving the equation for a bimolecular reaction 2M → D:

$$P_M = [(8KC + 1)^{1/2}\text{-}1]/4KC \qquad (2)$$

Where K is the equilibrium constant and C is the total peptide concentration. K was assumed to be temperature dependent according to the equation (3):

$$K = e^{-\Delta G/RT} \qquad (3)$$

The Gibbs–Helmholtz equation can be used to express the temperature dependence of ΔG in terms of ΔH_m and T_m as given by Equation (4):

$$\Delta G = \Delta H_m \,.\, (1 - T/T_m) + \Delta C_p \,.\, \{T - T_m - T \,.ln(T/T_m)\} \qquad (4)$$

Where ΔH_m is the enthalpy change at the melting temperature T_m, that is defined as the temperature at which $P_m = 0.5$. ΔC_p is the change in heat capacity that was initially assumed to be zero for the purpose of fitting because due to the high interdependence of ΔH_m and ΔC_p these parameters cannot be fitted simultaneously. Equations (1) through (4) were combined and the data fitted directly. ΔC_p was calculated afterwards from the dependence of ΔH_m from T_m and the standard free energy of unfolding ΔG_o (1 M standard state) was then calculated at T_o= 37 ^oC according to Equation (5):

$$\Delta G_o = \Delta H_m \,.\, (1 - T_o/T_m) + \Delta C_p \,.\, \{T_o - T_m - T_o \,.ln(T_o/T_m)\} - RT_o ln(C) \qquad (5)$$

Table 1: Thermodynamic parameters obtained from the temperature dependent circular dichroism analysis:

Peptide Complex	$\Delta H(T_m)$ (kcal mol^{-1})	$T_m(K)$	ΔG^o at 310 K (kcal mol^{-1})
P1P3	-30.1810	336.16	-8.7505
P1P4	-31.2353	325.53	-7.8886
P3P5	-32.8295	317.26	-7.1640
P4P5	-33.6369	314.74	-6.8996

6. Preparation of Peptide Liposome Hybrid Vesicle:[3]

Peptide liposome hybrid vesicles was prepared by the early reported method.[3] Breifly, egg yolk L-α-phosphatidylcholine (EYPC, 15.37 mg, 20 μmol) and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Amino(PolythyleneGlycol)2000](DSPE-PEG2000, 0.14 mg, 0.5 μmol) were dissolved in 1 mL DCM. Then 200 μL solution of P3/P5 (1:1, 15 μmol) was prepared by diluting their stock solution in MeOH. Both the organic solutions (DCM and MeOH) were mixed together in small 25 mL round bottom flask. After that thin and uniform lipid peptide bilayer was formed by slow evaporation of organic solvent on rota evaporator at 30 ^oC with 50 rpm rotations. Last traces of organic solvent were completely removed by applying high vacuum to the bilayer for 2 hrs. Then the bilayer thin flim was formed. After that the bilayer film was hydrated with 1 mL solution of 0.1 mM Proflavine hydrochloride solution in PBS buffer (10 mM phosphate, 150 mM NaCl, and pH 7.4). Hydration process was carried out for 1 hour with continuous agitation at room temperature and occasional sonication for each 15 mins (total time 180 s).Then the extra vesicular proflavine salt and the peptides were removed by size exclusion chromatographic separation using Sephadex G-25 column. Liposome containing aliquots were collected and small unilamellar liposomes were obtained after 20 times extrusion through 100 nm polycarbonate membrane using mini-extruder (Avanti Polar Lipids, Alabaster, AL). The unilamellar liposomes were again

subjected to Sephadex G-25 column to remove free drug liberated in the process of extrusion. Size, charge and morphology of liposomes were confirmed by using Zetasizer *NanoZS90* and FE-SEM and TEM respectively. Liposomes were stored at 8 ^{o}C for further use. By using the same protocol control liposome without coiled coil peptide was prepared.

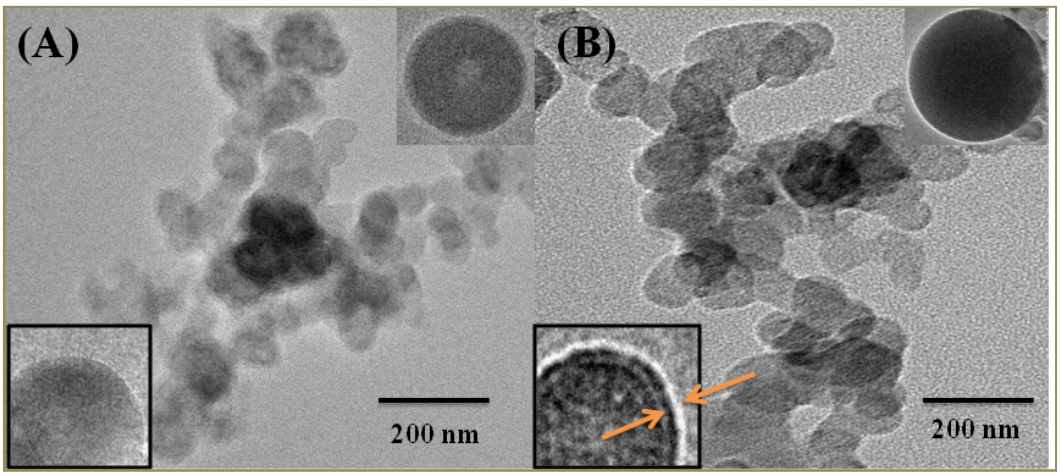


Figure S5: TEM images of the (A) peptide liposome hybrid vesicles and (B) control liposome without coiled coil peptide. Inserted image in the black box shows the bilayer nature of the liposome without coiled coil, whereas the bilayer signature was not present in coiled coil decorated liposome.

7. Stability of the Liposome:

Stability of the liposome was measured by the dynamic light scattering (DLS) experiment using 90^{o} scattering angle. The hydrodynamic diameter and PDI of the nanoparticle were measured from 1 to 14 days in PBS at 4 °C. The stability of the proflavine encapsulated peptide decorated liposome was further checked in DMEM containing 10% FBS.[4] The release of the encapsulated proflavine was monitored by the UV absorbance for 24 hrs. Percentage of the released proflavine was calculated from the standard curve.

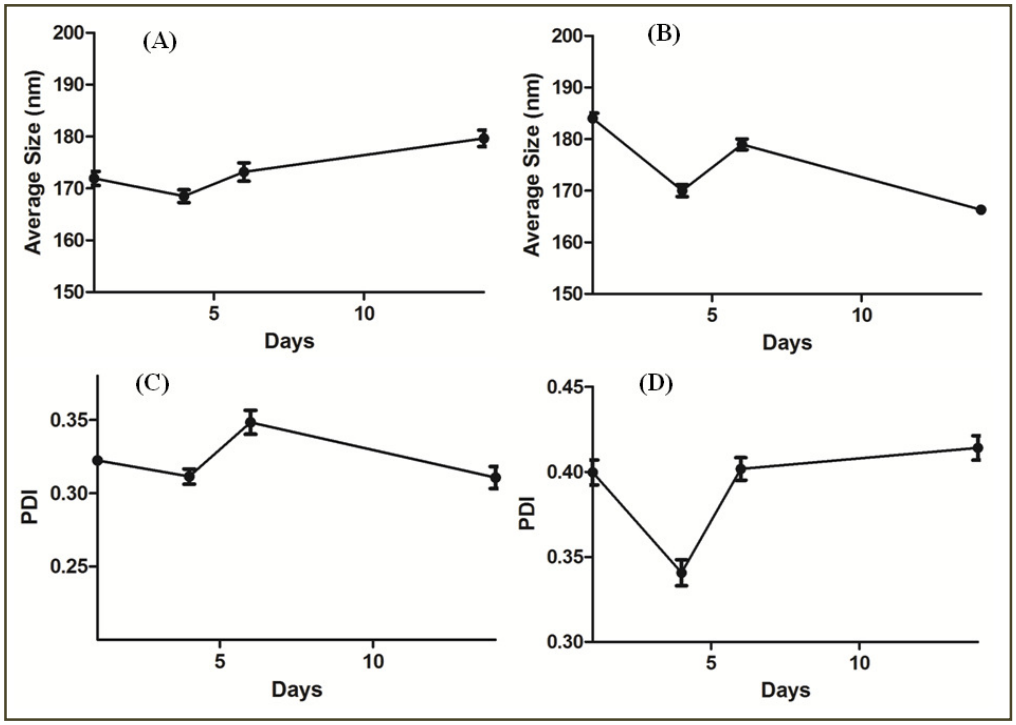


Figure S6: Stability of the liposome at 4 °C. Figures (A) and (C) are representing the peptide liposome hybrid vesicles, while (B) and (D) are representing the control liposome without coiled coil peptide.

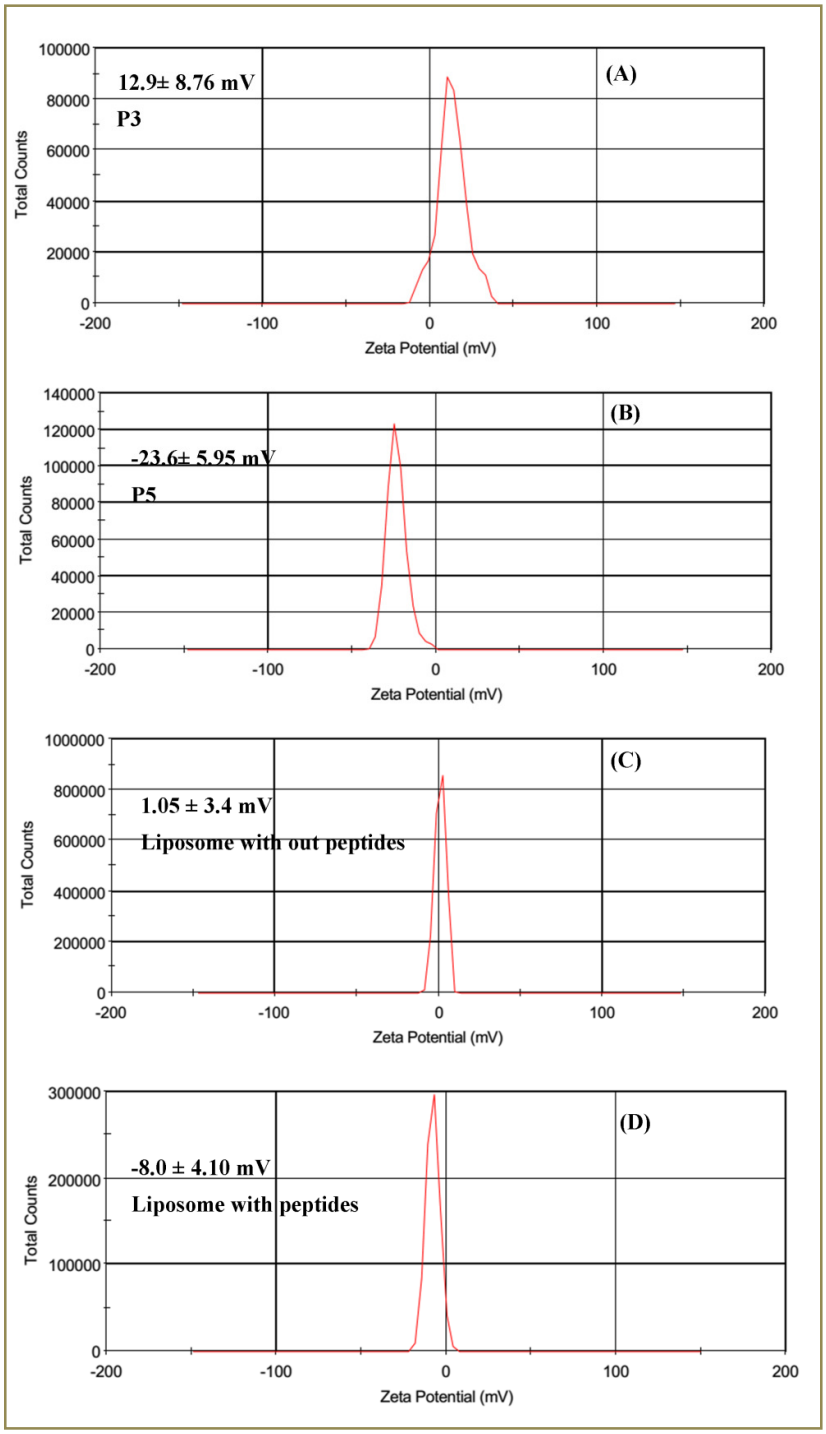


Figure S7: Zeta potential value of the (A) peptide **P3**, (B) peptide **P5**, (C) Liposome without peptides, (D) Liposome with peptides in PBS at pH 7.4. The conc. of the each peptide was 15 µM.

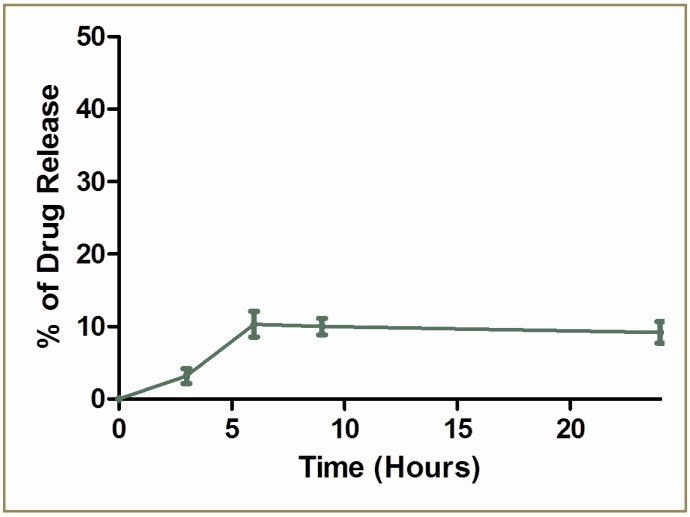
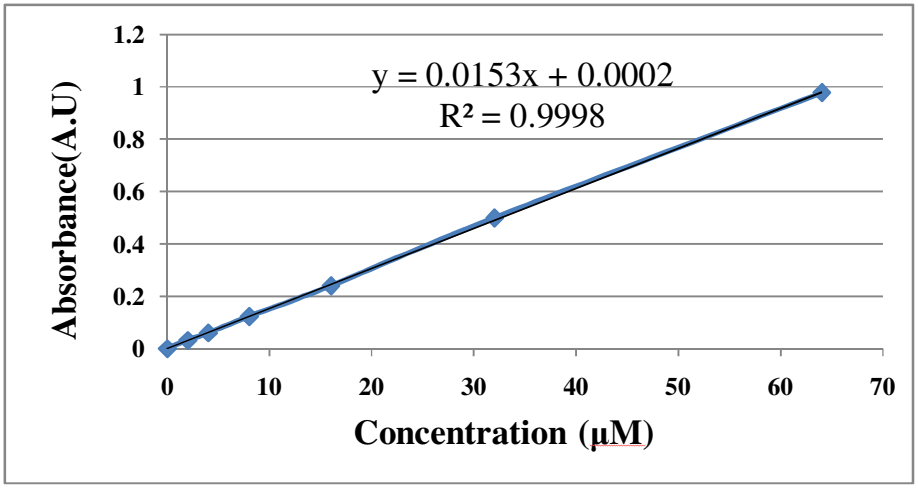


Figure S8: Stability of the proflavine encapsulated liposome in DMEM containing 10 % FBS. The percentage of the released proflavine with respect to time is shown.

8. Procedure for pH Triggered Percentage of Drug Release:

Lipid peptide hybrid liposome (200 µL) loaded with proflavine hydrochloride was sealed in dialysis membrane having molecular weight cut off 500 Da. This dialysis bag was suspended in agitating PBS buffer (3 mL, 10 mM phosphate, 150 mM NaCl, and pH 7.4) and acetate buffer (3 mL, 10 mM acetate buffer at pH 5.5), at the 37 °C, separately. Control studies also done by taking the liposome without coiled coil peptides. 200 µL aliquot from the suspension medium was timely collected and diluted to 400 µL with respective buffer solution. Then quantification of released proflavine was carried out by UV/Vis spectrometer. The percentage of released drug was calculated from corresponding calibration curve for the proflavine hydrochloride in the buffer solution.



\

Figure S9: Calibration curve for the quantification of the percentage of the drug release by the UV measurement.

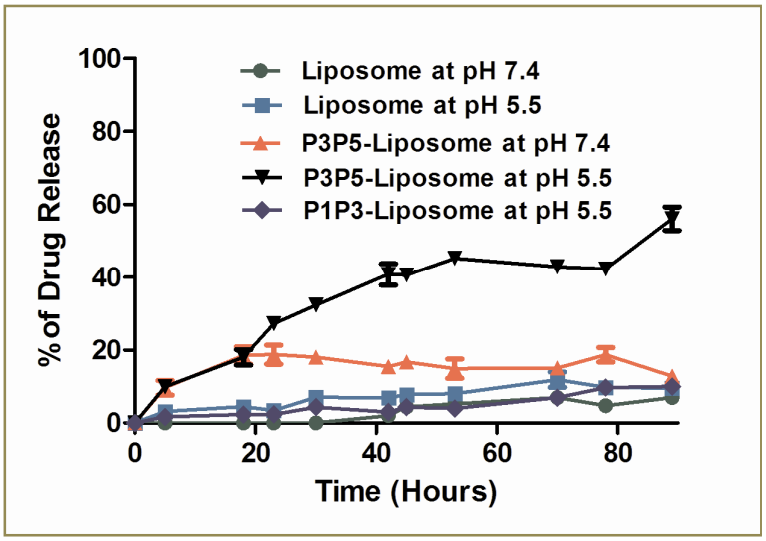


Figure S10: Release kinetics profile of the **P3P5**-Liposome and **P1P3**-Liposome and Liposome without peptide in different pH.

9. Procedure for MTT Assay in LN229 Cell Line:

LN229 cells were seeded in 96 well plate at a density of 5000 cells per well in 200µ L of DMEM medium supplemented with 5% FBS and allowed to attain morphology overnight at 37 °C and 5% CO_2 incubator. Cells were treated with different concentration of free drug and drug encapsulated peptide liposome hybrid vesicles with equivalent amount of drug for 48 hrs. After 48 hrs 20 µL of MTT (5 µg/µL) reagent was added to the cells and allowed to form formazan crystals for 4 hrs at 37 °C and 5% CO_2. After 4hrs media was discarded without disturbing the formazan crystals. Crystal formed by the viable cells were dissolved in DMSO and quantified by measuring the O.D. at 570 nm with Elisa reader. Percent survival of cells on treatment with drug only and drug encapsulated peptide hybrid liposome was calculated by comparing the amount of formazan crystal formed with untreated cells.

10. Procedure for the Cell Internalization in LN229 Cell Line:

2 x 10^4 LN229 cells were seeded on coverslip in 24 well plate incubated at 37 °C& 5% CO_2 to allow them to attain their morphology. Cells were treated with 0.2 µM free proflavine and peptide liposome hybrid encapsulated with 0.2 µM proflavine for 1h, 3h and 7h. Cells were also treated with Lyso Tracker DND 99 (Life Technology) for 30 minutes. After specific time intervals cells were washed thrice with PBS and fixed for 15 minutes with 4% PFA at room temperature. Cells were again washed thrice with PBS for 5 minutes each. To stain nucleus cells were incubated with 2 mg/mL Hoechst for 15 minutes at room temperature. To remove unbound Hoechst cells were washed three times with PBS and mounted on glass slide with mounting medium. Imaging was performed with Olympus Fluoview confocal microscope. Lyso Tracker, Hoechst and proflavine were excited at 577 nm, 405 nm and 444 nm respectively.

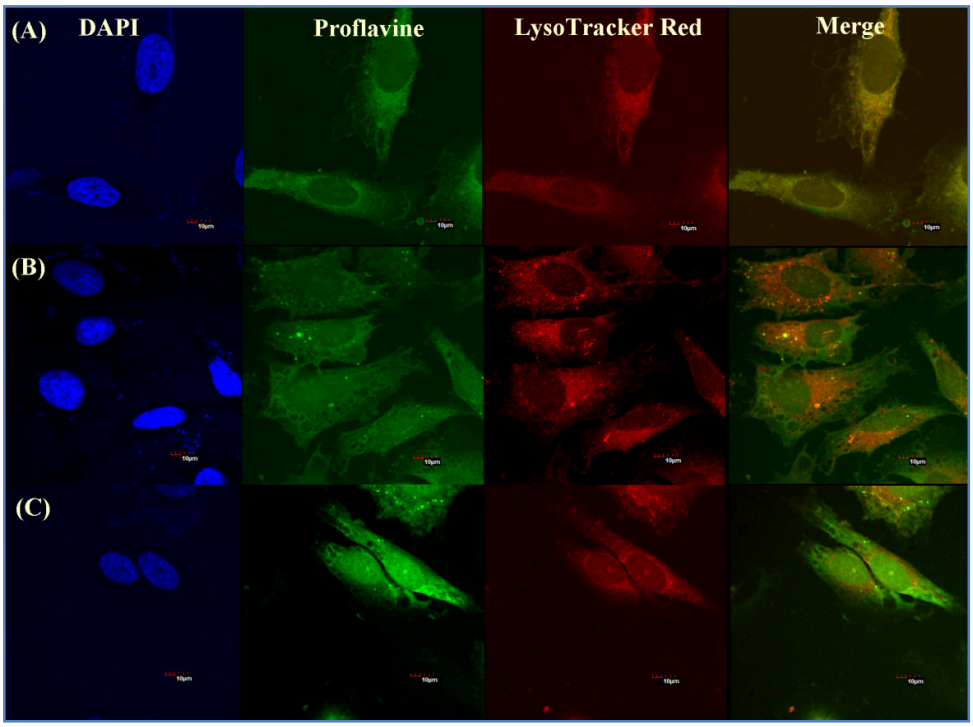


Figure S11: Confocal Laser Scanning Microscopy (CLSM) images of the internalization of the free Proflavine in LN 229 cell lines at different time interval time. The images (A), (B) and (C) are indicating the time 1hr, 3 hrs and 7 hrs, respectively. Low pH lysosomal compartment and nucleus are strained by the LysoTracker Red DND 99 and DAPI (Scale : 10 μm).

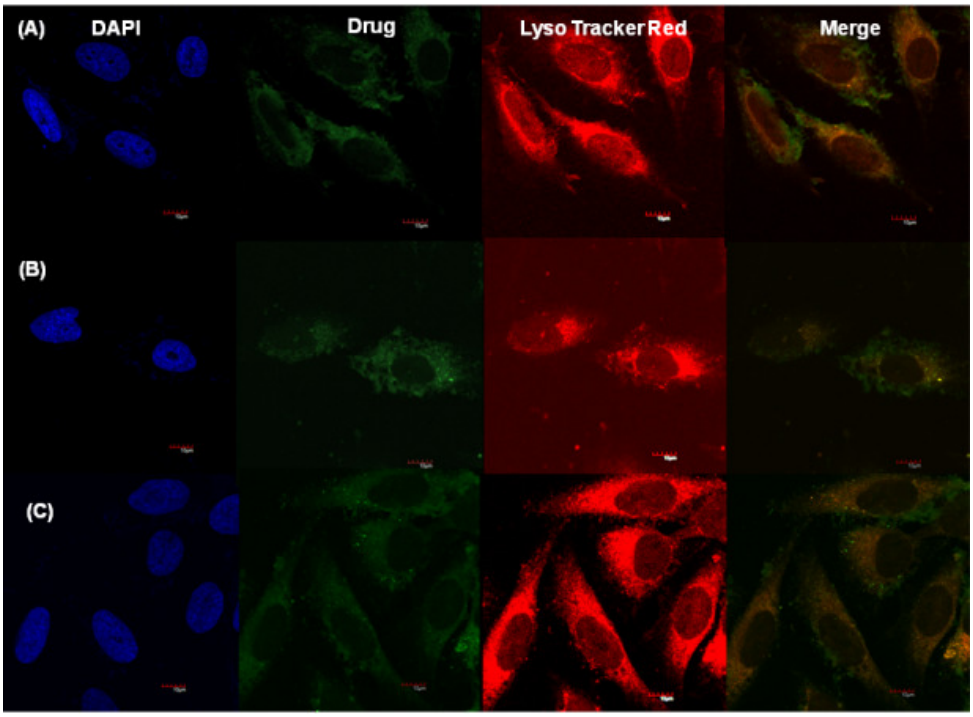


Figure S12: Confocal Laser Scanning Microscopy (CLSM) images of the internalization of the Liposome without peptide in LN 229 cell lines at different time interval time. The images (A), (B) and (C) are indicating the time 1hr, 3 hrs and 7 hrs, respectively. Low pH lysosomal compartment and nucleus are strained by the LysoTracker Red DND 99 and DAPI (Scale : 10 μm).

11. Calculation of Percentage of Vol. Colocalization:[5]

Percentage of the vol. colocalization was calculated by the Image J software with the help of the GDSC Colocalization threshold from their raw image file.

Table 2 : Percentage of vol. colocalization of the P3P5-Liposome hybrid vesicle

Treatment of time	**1hr**	**3hrs**	**7hrs**
Pearsons' Correlation Coefficient	**0.7075**	**0.7248**	**0.6854**
Manders Coefficients			
M1 (fraction of C2 overlapping C3)	**0.7783**	**0.8139**	**0.8923**
M2 (fraction of C3 overlapping C2)	**0.7833**	**0.7859**	**0.9403**
Percent volume colocalized	**44.07**	**60.46**	**90.79**

C2 channel is green channel and C3 is red channel.

Table 3 : Percentage of vol. colocalization of the P1P3-Liposome hybrid vesicle

Treatment of time	**1hr**	**3hrs**	**7hrs**
Pearsons' Correlation Coefficient	**0.7413**	**0.6382**	**0.611**
Manders Coefficients			
M1 (fraction of C2 overlapping C3)	**0.3786**	**0.677**	**0.5881**
M2 (fraction of C3 overlapping C2)	**0.9444**	**0.9461**	**0.8867**
Percent volume colocalized	**12.71**	**26.34**	**49.03**

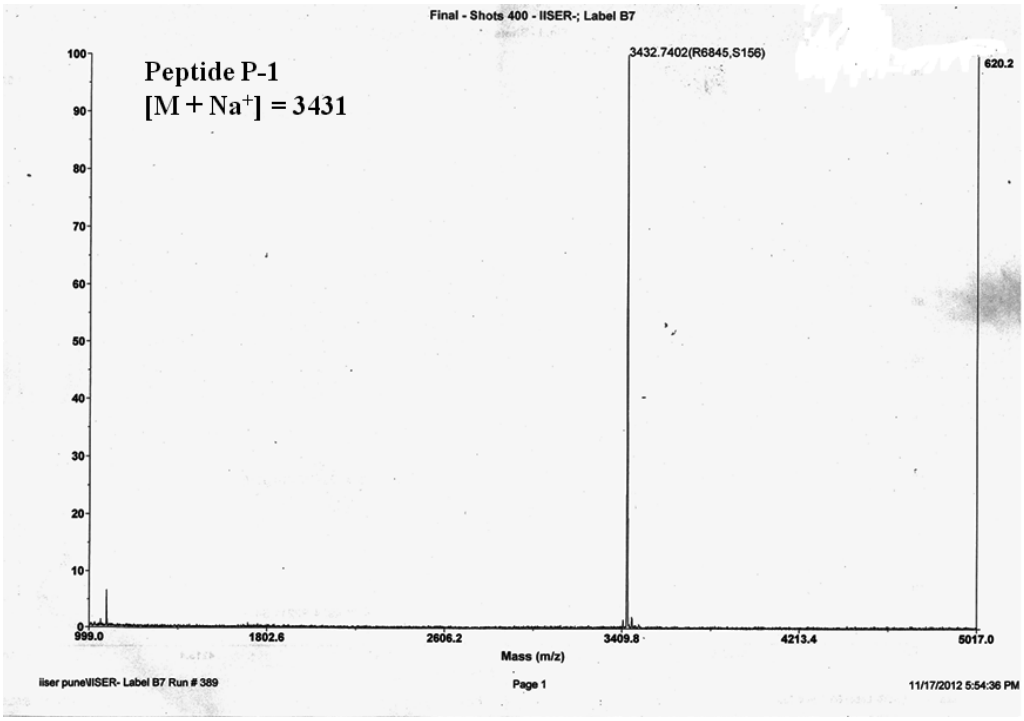
C2 channel is green channel and C3 is red channel.

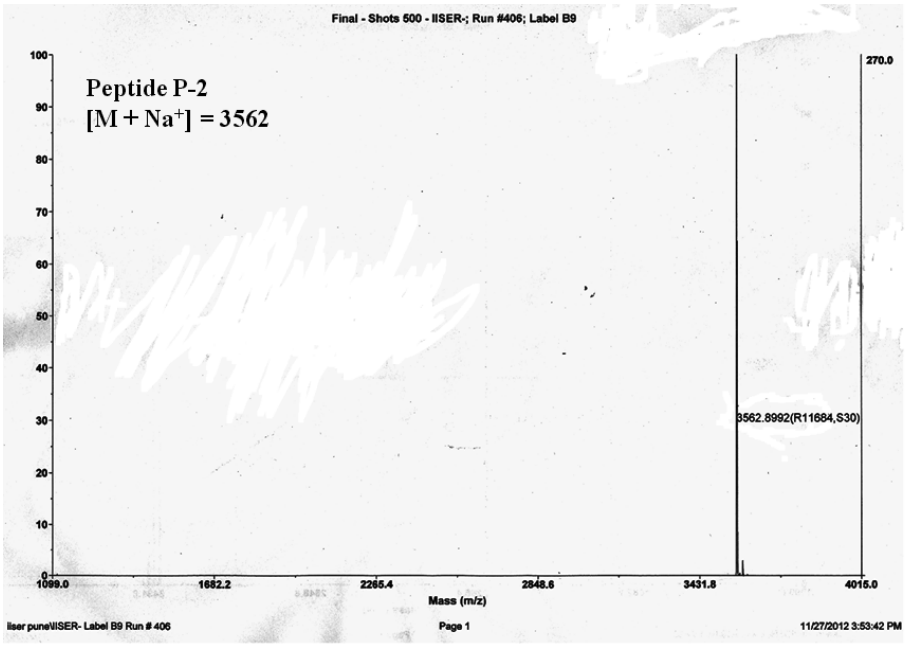
Table 4 : Percentage of vol. colocalization of the liposome without peptides

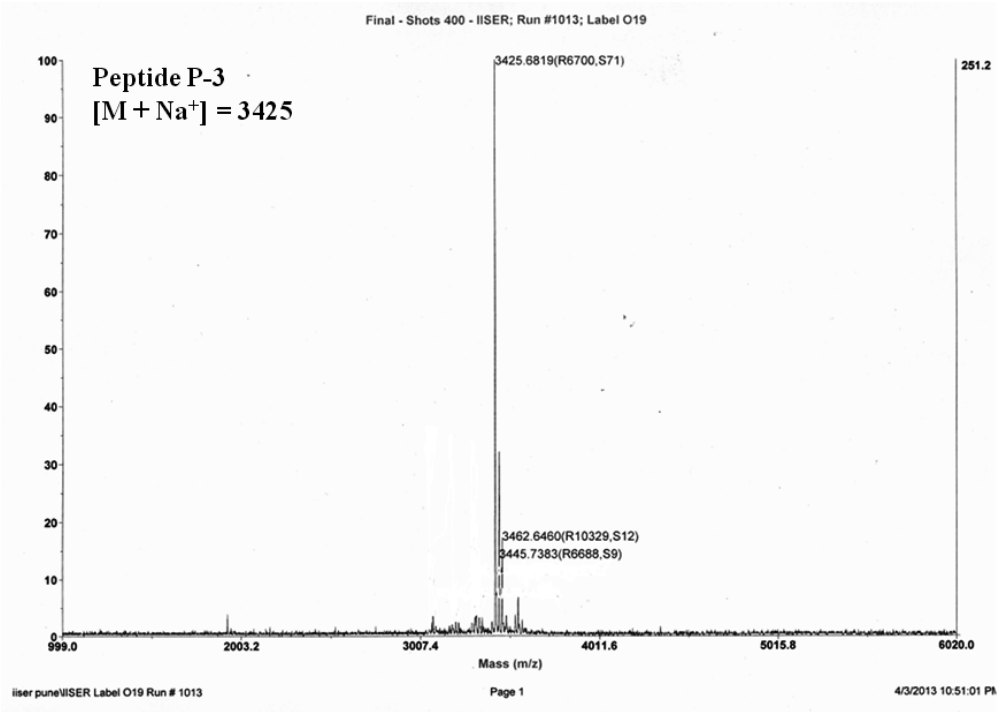
Treatment of time	1hr	3hrs	7hrs
Pearsons' Correlation Coefficient	**0.6638**	**0.7744**	**0.7536**
Manders Coefficients			
M1 (fraction of C2 overlapping C3)	**0.6281**	**0.4921**	**0.8185**
M2 (fraction of C3 overlapping C2)	**0.5858**	**0.4495**	**0.8177**
Percent volume colocalized	**23.61**	**37.19**	**62.97**

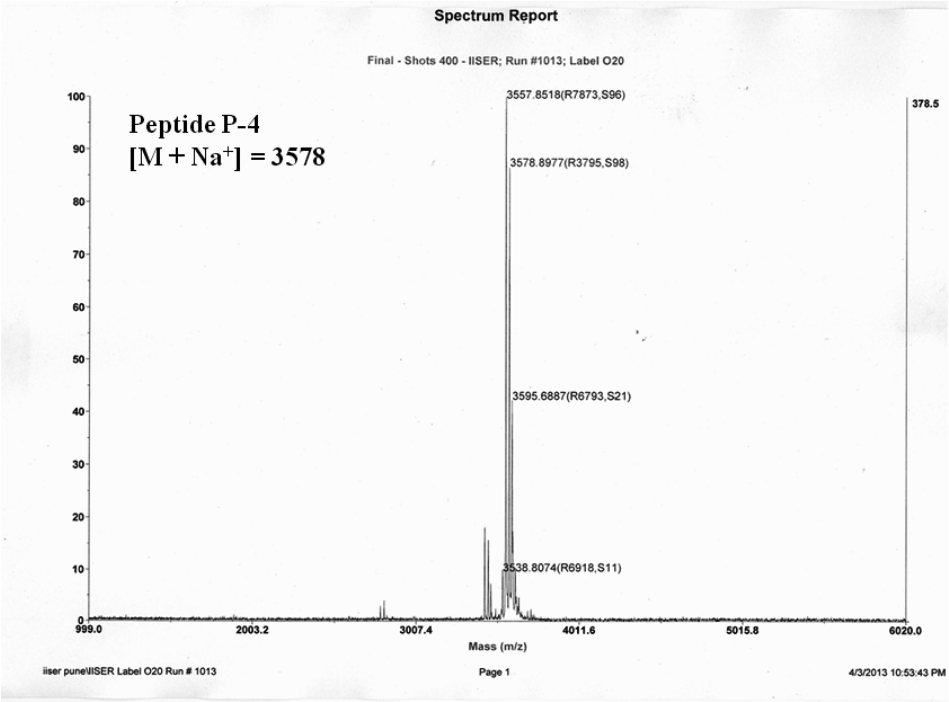
C2 channel is green channel and C3 is red channel.

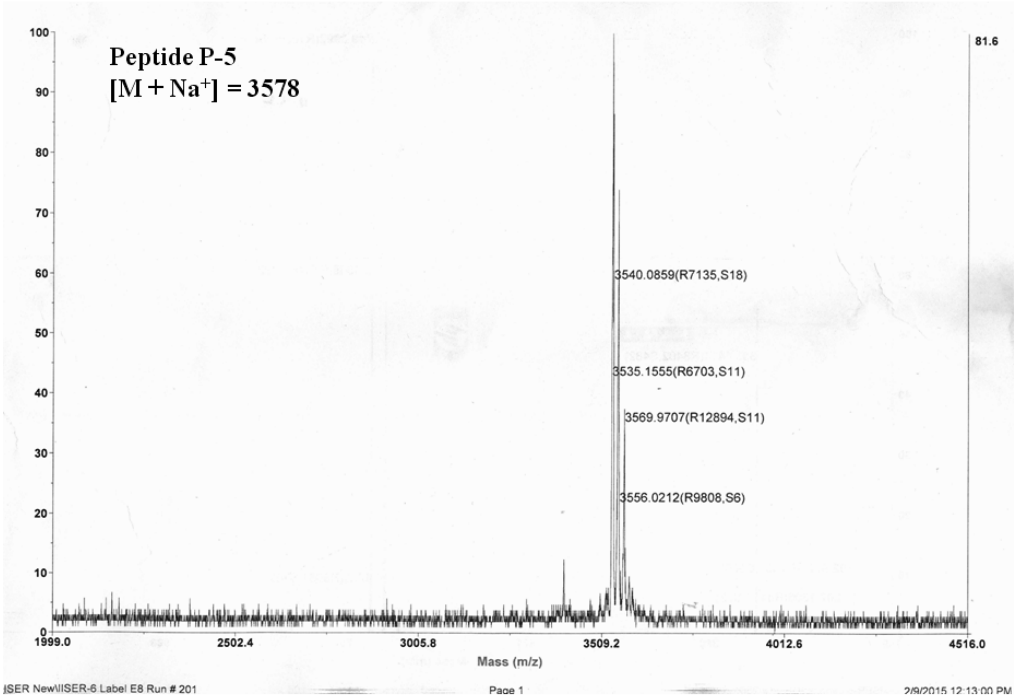
12. MALDI-TOF/TOF spectra of Peptides :



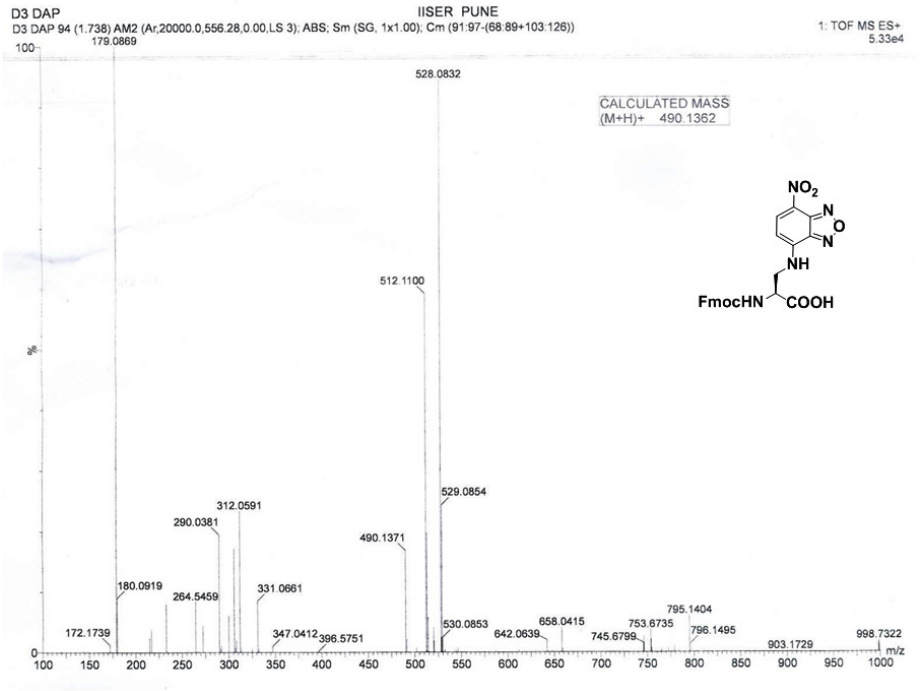



Final - Shots 400 - IISER; Run #1013; Label O19
Peptide P-3
[M + Na+] = 3425
3425.6819(R6700,S71)
251.2
3462.6460(R10329,S12)
3445.7383(R6688,S9)
100
90
80
70
60
50
40
30
20
10
0
999.0
2003.2
3007.4
4011.6
5015.8
6020.0
Mass (m/z)
iiser pune\IISER Label O19 Run # 1013
Page 1
4/3/2013 10:51:01 PM

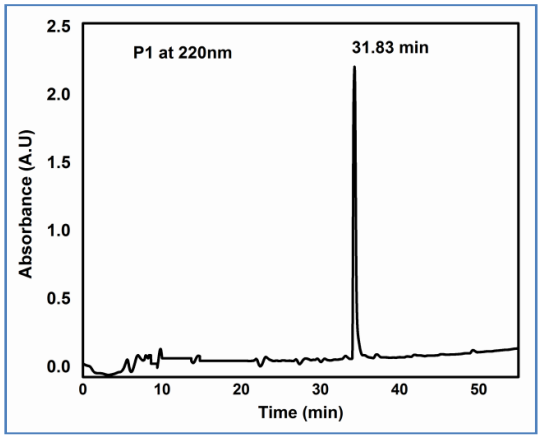

Spectrum Report
Final - Shots 400 - IISER; Run #1013; Label O20
Peptide P-4
[M + Na+] = 3578
3557.8518(R7873,S96)
378.5
3578.8977(R3795,S98)
3595.6887(R6793,S21)
3538.8074(R6918,S11)
100
90
80
70
60
50
40
30
20
10
0
999.0
2003.2
3007.4
4011.6
5015.8
6020.0
Mass (m/z)
iiser pune\IISER Label O20 Run # 1013
Page 1
4/3/2013 10:53:43 PM

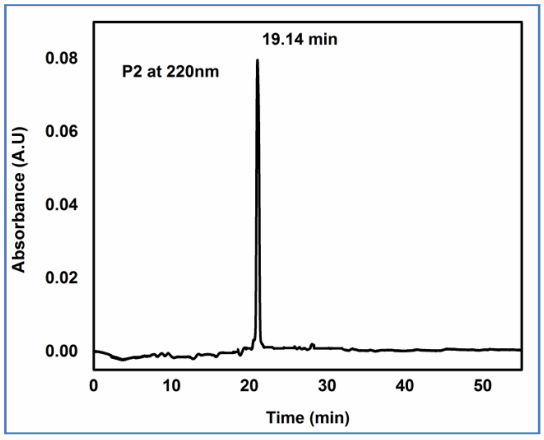


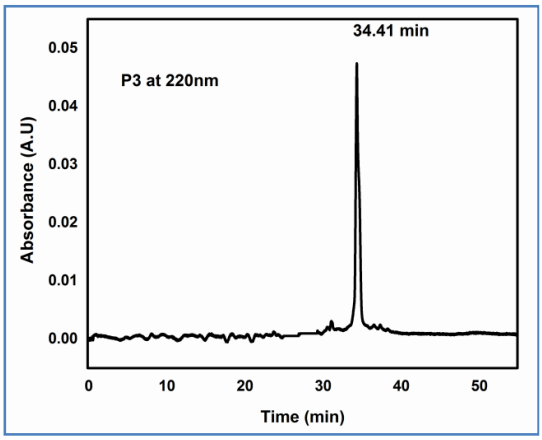
13. HRMS Spectrum of the NBD Derivative Monomer:

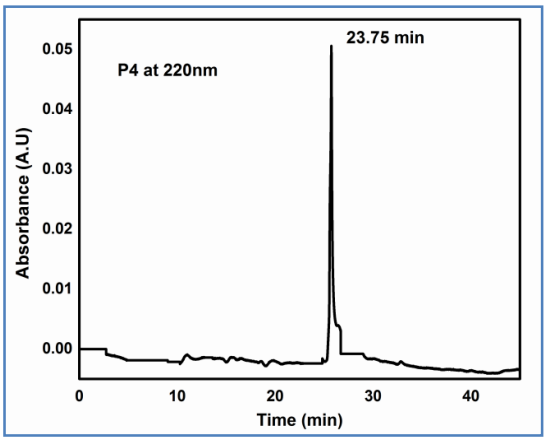


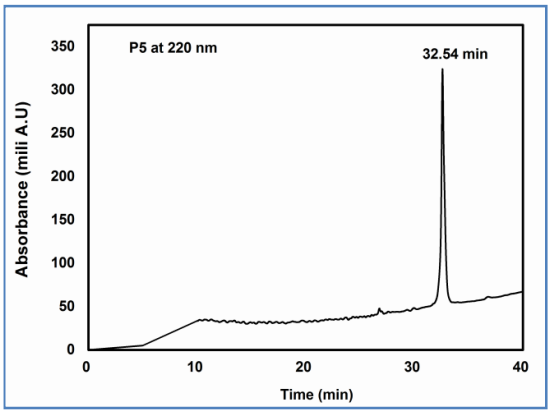
14. HPLC Traces of the Pure Peptides:





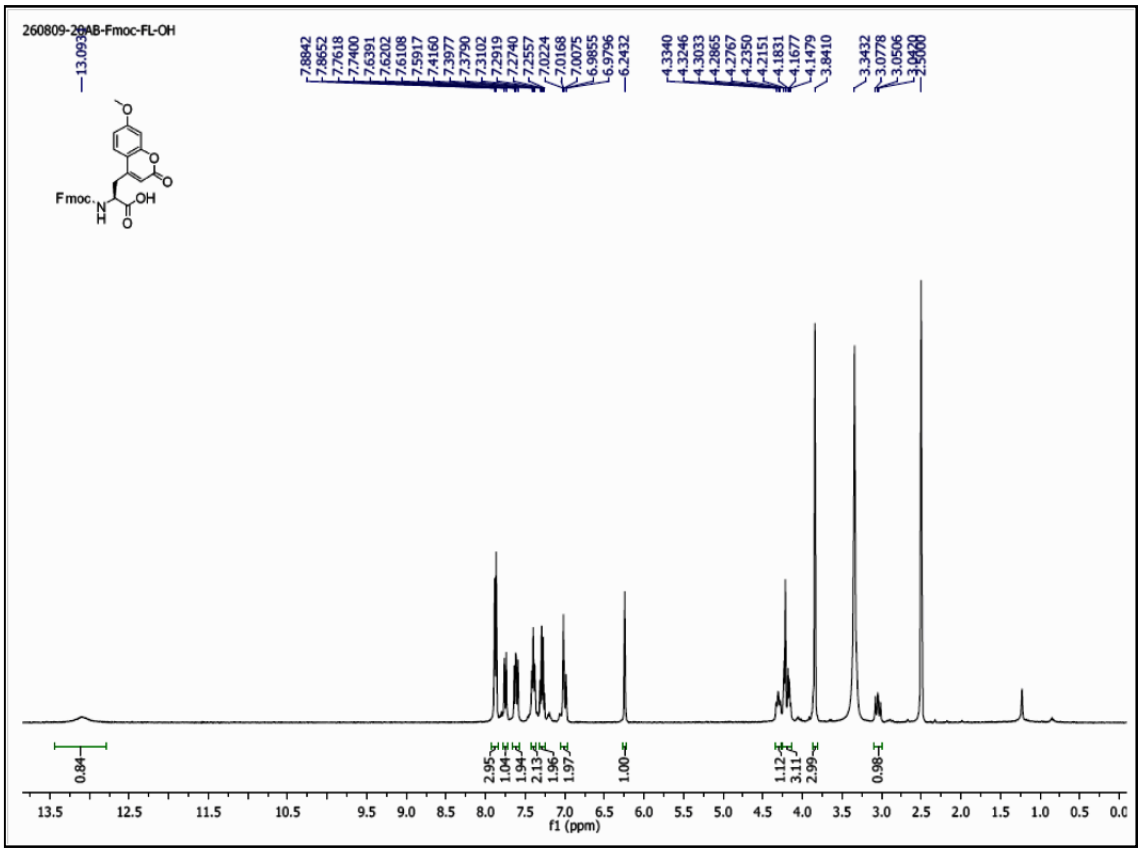




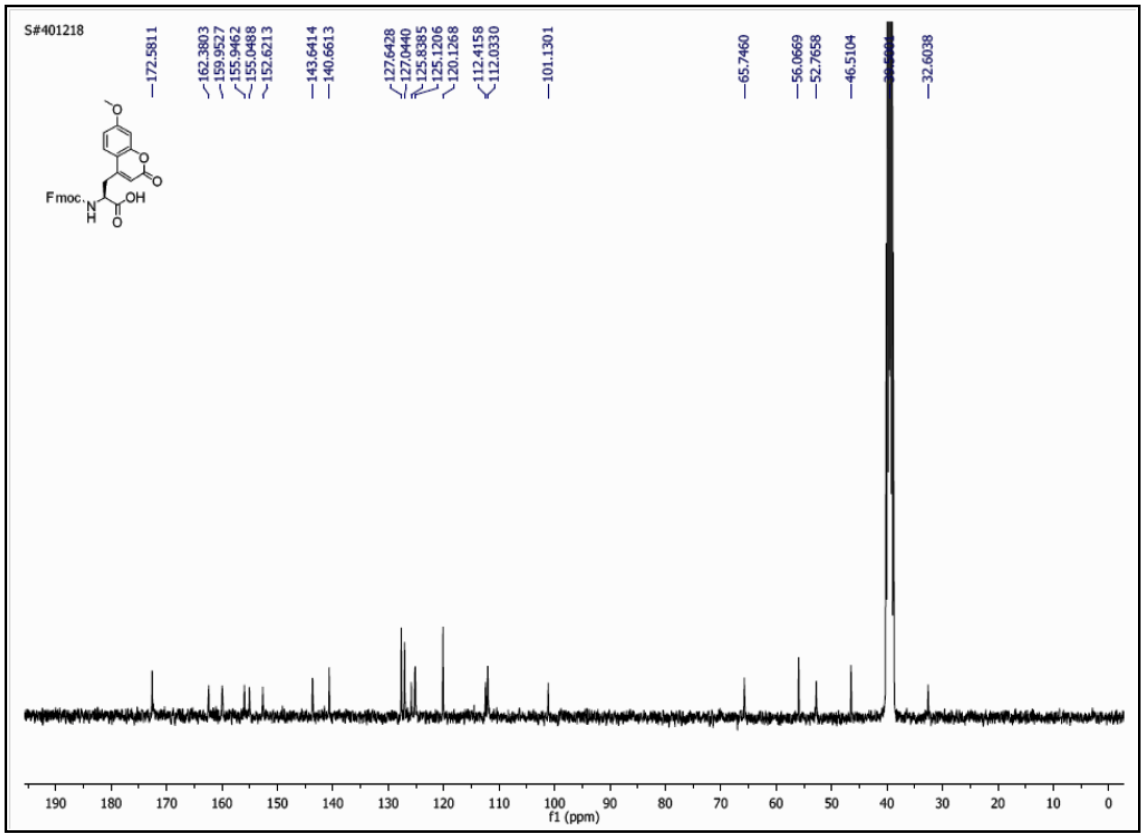


15. NMR Spectrum:

^{1}H NMR



^{13}C NMR



16. References:

1. A. Bandyopadhyay and H. N. Gopi, *Org. Biomol. Chem.*, 2011, **9**, 8089.
2. I. Dufau and H. Mazarguil, *Tetrahedron Lett.*, 2000, **41**, 6063.
3. S. V. Jadhav, S. K. Singh, R. M. Reja and H. N. Gopi, *Chem. Commun.*, 2013, **49**, 11065.
4. K. Vogel, S. Wang, R. J. Lee, J. Chmielewski and P. S. Low, *J. Am. Chem. Soc.*, 1996, **118**, 1581.
5. K. W. Dunn, M. M. Kamocka and J. H. McDonald, *Am. J. Physiol. Cell Physiol.,* 2011, **300**, C723.